

SSeCKS is a Suppressor in Schwann Cell Differentiation and Myelination

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Abstract Src-suppressed protein kinase C substrate (SSeCKS) plays an important role in the differentiation process. In regeneration of sciatic nerve injury, expression of SSeCKS decreases, mainly in Schwann cells. However, the function of SSeCKS in Schwann cells differentiation remains unclear. We observed that SSeCKS was decreased in differentiated Schwann cells. In long-term SSeCKS-reduced Schwann cells, cell morphology changed and myelin gene expression induced by cAMP was accelerated. Myelination was also enhanced in SSeCKS-suppressed Schwann cells co-culture with dorsal root ganglion (DRG). In addition, we found suppression of SSeCKS expression promoted Akt serine 473 phosphorylation in cAMP-treated Schwann cells. In summary, our data indicated that SSeCKS was a negative regulator of myelinating glia differentiation.

Keywords Schwann cell · Differentiation · Myelination · SSeCKS · Akt

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Introduction

Schwann cells, myelin-forming cells in the peripheral nervous system (PNS), arise from the neural crest, then lose the mitotic activity and form a 1:1 relationship with axons. Promyelinating Schwann cells wrap around axon in a radial growth and migration process, establishing multiple concentric membrane layers, which are lipid and protein rich layers to support and electrically insulate them [1, 2]. The differentiation and myelination program during peripheral nerve development is primary dependent on the expression of two transcription factors, Oct6/Scip/Tst-1 and Krox20/Egr2 [3–5]. This process is accompanying with specific genes expressions such as myelin-associated glycoprotein (MAG), Protein Zero (P0), Lgi4 and cAMP-inducible POU. Immature Schwann cells markers such as glial fibrillary acidic protein and NGFRp75 are reduced [6, 7].

The molecular cues regulating this complicated choreography are unknown. Several groups reported that the activity of extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (Akt or PKB) were involved in myelination [8]. One signal that can mimic axonal contact with Schwann cells is cyclic adenosine monophosphate (cAMP). Originally recognized for its ability to up-regulate the myelin marker galactocerebroside in Schwann cells [9], cAMP was subsequently shown to increase the expression of a number of myelin genes such as P0 [10, 11] and the transcription factor Oct6/Scip/Tst-1 [3–5]. Protein kinase A (PKA), the well-known down stream effector of cAMP, is activated on the nucleotide binding to the regulatory subunit of the kinase. Inhibition of PKA was shown to block the morphological changes in Schwann cells induced by elevation of cAMP level with forskolin and prevent the formation of myelin in Schwann cells-dorsal root ganglia (DRG) neuron co-cultures [12]. However, the critical

targets of PKA in promoting Schwann cells differentiation into the myelinating phenotype have yet to be identified.

Src-suppressed protein kinase C substrate (SSeCKS) was originally identified in the screening for genes severely down-regulated by *v-Src* [13] and characterized as a major in vitro and in vivo substrate of protein kinase C (PKC) [14]. SSeCKS is a rodent orthologue of human gravin, a kinase scaffold protein discovered as an autoantigen in some myasthenia gravis patients [15, 16]. Based on their ability to bind PKA regulated II isoforms [15], SSeCKS and gravin have been re-designated as kinase anchoring protein 12 (AKAP12). In addition to PKA and PKC, SSeCKS also binds with calmodulin, cyclin D1, β -adrenergic receptor, β -1,4 galactosyltransferase and F-actin [17]. With its ability to scaffold key signaling and cytoskeleton proteins, SSeCKS play an important role in regulating the change of morphological in various types of cells, including endothelial and HEK 293T cells [18, 19]. It has been reported that during the late-stage of spermatogenesis, SSeCKS was increased in the beginning 30 days after birth [20]. Our own group has also found that SSeCKS was detected in whole brain of developing rat embryos and reached its peak at 1 week after birth, while during mature period, its level was decreased [21]. We have confirmed that SSeCKS reached its peak level at the early stage of peripheral nerve injury, and then decreased rapidly in the regeneration progress when Schwann cells differentiated and formed myelination [22]. Whether SSeCKS is involved in the Schwann cells differentiation and its role in myelination have not been reported.

In our study, we showed that SSeCKS was a negative regulator of Schwann cells differentiation affecting a number of important processes such as morphology and myelin expression. SSeCKS inhibited the phosphorylation of Akt at serine 473, and prevented myelinating progress. These results indicated a molecular mechanism which was important for myelinating glial cell differentiation as well as the establishment of myelin sheaths.

Experimental Procedures

Primary Schwann Cells Cultures

Primary rat Schwann cells were isolated from sciatic nerves of 1 to 3 days old Sprague Dawley rats. The cells were expanded on collagen (type I from rat tail; Sigma)-coated plates in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovin serum (FBS; Sigma) and forskolin (2 μ M; Sigma). For purification, the cells were treated with cytosine arabinoside (10 μ M; Sigma) twice for 24 h and subjected to immunopanning with antibody against Thy1.1 (Sigma). For

differentiation experiments, the purified cells were cultured in DMEM supplemented with 10% FBS for 3 days. After that, these cells were cultured in serum-free, defined media, which consisted of a 1:1 mixture of DMEM supplemented with 10% FBS and Ham's F-12 with N2 supplement (Invitrogen), for additional 2 days before treatment with cAMP (10 μ M; Sigma), and/or LY294002 (10 μ M; Cell Signal Technology).

DRG and Schwann Cells Co-Culture

For cultures of pure rat DRG neurons, to be seeded with Schwann cells, the DRGs which isolated from E15 rats were dissociated and plated at a density of 30,000 cells/2.2 cm² coverslip in serum-free Essential Modified Eagle's Medium (EMEM; Invitrogen) containing 50 ng/ml nerve growth factor (NGF; Sigma). The cells were then treated twice for 48 h with uridine (10 μ M; Sigma) and fluorodeoxyuridine (10 μ M; Sigma) to remove all non-neuronal cells. The neurons and Schwann cells were co-cultured in EMEM supplemented with 10% FBS, and 50 ng/ml NGF at a density of 70,000 cells/2.2 cm² collagen-coated cover slip. Myelination was studied 5 days later by adding 50 μ g/ml ascorbic acid (Sigma), and the cultures were maintained by replacing with fresh media containing ascorbic acid every other day.

Generation of Lenti-SSeCKS siRNA and Transfection of Schwann Cells

The rat SSeCKS siRNA nucleotide sequences: Top strand, 5'-TGCTGTTTCGATTGCTGTACTCTCCTTGTTTTGGCCACTGACTGACAAGGAGAGCAGCAATCGAA-3'; Bottom strand: 5'-CCTGTTCGATTGCTGCTCTCCTTGTCAGTCAGTG directly cloned into the pLenti6/BLOCK-iT-DEST plasmid (Invitrogen) according to manufacturer's instructions. 3 μ g of pLenti6/BLOCK-iT-DEST EGFP-siRNA vector and 9 μ g of ViraPower Packaging Mix (Invitrogen) were used to co-transfect 5×10^6 293FT cells with Lipofectamine 2000. 48 h after transfection, the supernatant containing the viruses (Lenti-EGFP-SSeCKS siRNA) was harvested, centrifuged and stored at -80°C . Schwann cells were transfected with Lentivirus-EGFP-SSeCKS siRNA using a multiplicity of infection for 48 h and then proceeded for fluorescence and RT-PCR to determine the transfection efficiency.

RNA Preparation and RT-PCR

Total RNA were extracted from Schwann cells using Trizol reagent as described by the manufacturer (Invitrogen). Normally 500 ng of total RNA were reverse-transcribed into cDNA using the ThermoScript RT system (Fermentas).

The following primers were used: SSeCKS: forward, 5'-AA GAATGGCCAGCTGTCTAC-3'; reverse, 5'-GCTTTGGA ACTGTCTGTCACT-3'; MAG, forward, 5'-ACTGGTGT GTAGCTGAGAAC-3'; reverse, 5'-GACAATGGCAATC AGGATGG-3'; P0, forward, 5'-GCTCTTCTCTTCTTTG GTGCTGTCC-3'; reverse, 5'-GGCGTCTGCCGCCCGCG CTTCC-3'; GAPDH: forward, 5'-TGATGACATCAAGA AGGTGGTGAAG-3'; reverse, 5'-TCCTTGAGGCCAT GTGGGCCAT-3'. PCR amplification was carried out with an initial denaturing step at 94°C for 5 min, then 30 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 45 s, followed with a further extension at 72°C for 10 min. The PCR products were electrophoresed through a 1% agarose gel, and visualized by ethidium bromide staining. The relative differences of the expression between groups were normalized with GAPDH.

Immunoblot Analysis

Cultured Schwann cells and co-cultured cells were collected and lysed with SDS sample buffer. All the samples were quantitated using protein assay kit (Bio Rad). Equal amounts of total protein were separated by SDS polyacrylamide gel electrophoresis and electrophoretic transferred to PVDF membrane. Followed that membrane were immunoblotted with primary polyclonal antibodies and monoclonal antibodies including SSeCKS, GAPDH, Akt, p-Akt^{S473}. Following three Tris-buffered saline Tween 20 (TBST) washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. After three times of TBST washes, the secondary antibodies were visualized using LumiGLO Regent and Peroxide (Cell Signal). The optical density on the film was measured with a computer imaging system (Imaging Technology, Ontario, Canada). The relative difference between the control and treatment groups was calculated and expressed as a relative ratio to the control. The control was set as 1. Values were represented for three independent assays.

Immunofluorescence Staining

Cells were fixed at 4°C for 20 min with precooled PBS that contained 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 min, and then blocked by 1% BSA for 2 h. After washing in PBS, the cells were incubated for 1 h with sheep polyclonal anti-SSeCKS antibody at 1:250 dilutions. Cells were washed with PBS 5 times and incubated with TRITC-conjugated anti-sheep IgG for 30 min. At last the cells were washed with PBS and reversed on glass slides with glycerol and PBS (1:1). The cells were examined under a Leica Confocal Laser Scanning Microscope and fluorescence microscope.

Statistical Analysis

All experiments were repeated at least three times. All numerical data are described as mean \pm SD. Data was analyzed using the two-tailed *t* test. A probability value of 0.05 or less was considered significant.

Results

SSeCKS Expression is Down-Regulated During Schwann Cells Differentiation

Previous study of our groups showed that SSeCKS was detectable in healthy adult rat sciatic nerves, and its expression was reduced during the repair process after injury [22]. When treated Schwann cells with 10 μ M cAMP, we confirmed that SSeCKS expression was significantly down-regulated after 7 days treatment (Fig. 1a), and Schwann cells became polygonal, longer, fine, and tapering in the differentiation process (Fig. 1b). These data indicated that SSeCKS might play a negative role in Schwann cells differentiation.

Long-Term Suppression of SSeCKS Expression Enhances cAMP-Induced Morphological Changes

To examine the role of SSeCKs in Schwann cells differentiation, we knocked down SSeCKS expression in Schwann cells by application of EGFP-SSeCKS siRNA lentivirus. SSeCKS mRNA expression levels, determined by RT-PCR analysis, were reduced about 80% (Fig. 2a). Anti-SSeCKS immunofluorescent staining of SSeCKS-suppressed Schwann cells that have been labeled by means of EGFP expression vector confirmed the reduced SSeCKS protein expression (Fig. 2b).

We further investigated whether the SSeCKS suppression also affected the differentiation-associated processes, such as cell morphology changes. We observed that after 10 μ M cAMP treatment, SSeCKS-suppressed Schwann cells began to be polygonal, somata and formed cellular extensions at day 3. However, control lenti-EGFP vector-transfected Schwann cells did not change their morphology until day 5 (Fig. 2b).

Myelin-Associated Gene Expressions are Enhanced in SSeCKS-Suppressed Schwann Cells

When Schwann cells wrap around axons, they express myelin gene and present myelin protein within the layers of wraps. Therefore we determined the myelin and myelin-associated gene levels such as P0 and MAG in the differentiation process. P0 and MAG were significantly induced

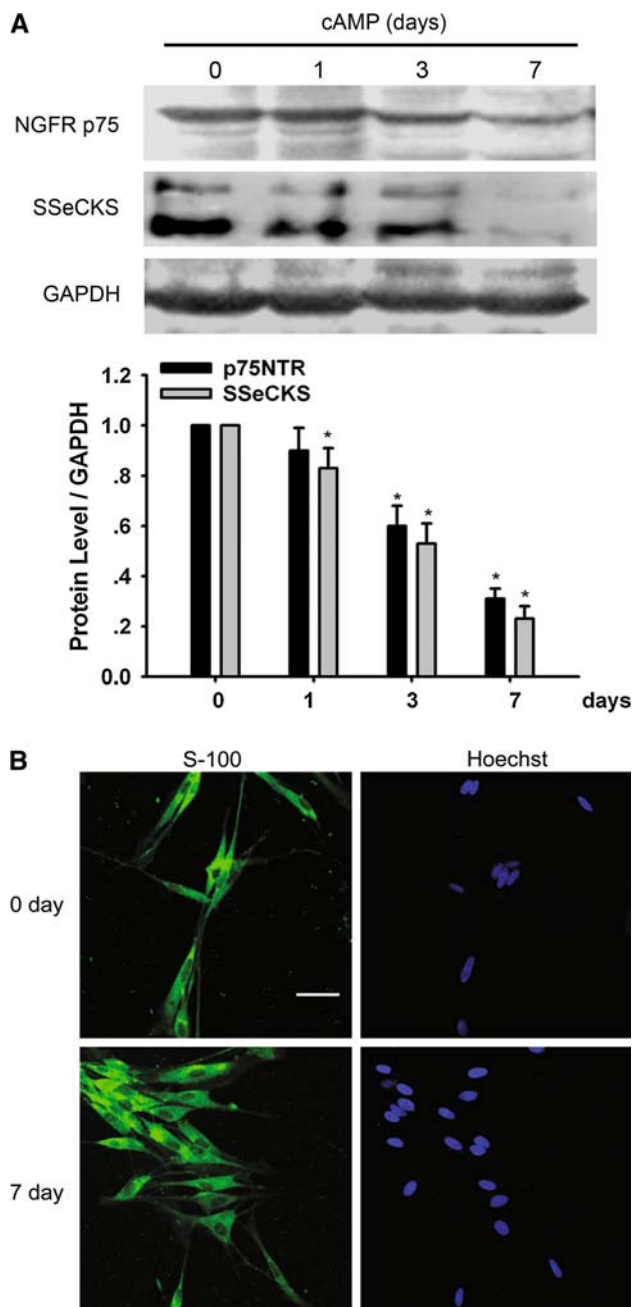


Fig. 1 SSeCKS expression is down-regulated in Schwann cells differentiation process. **a** Western blot analysis revealed that by 10 μ M cAMP treatments, the undifferentiated marker NGFRp75 expression were decreased, which indicated Schwann cells differentiation. SSeCKS expression was down-regulated in differentiation process. GAPDH expression was used as a reference, and data are mean values \pm SEM. *t* test demonstrated significant difference (* $P < 0.001$, $n = 3$). **b** Immunofluorescence with S-100 (Schwann cell mark, green) showed that morphology and size were changed in cAMP-treated Schwann cells. Scale bars: 20 μ m: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

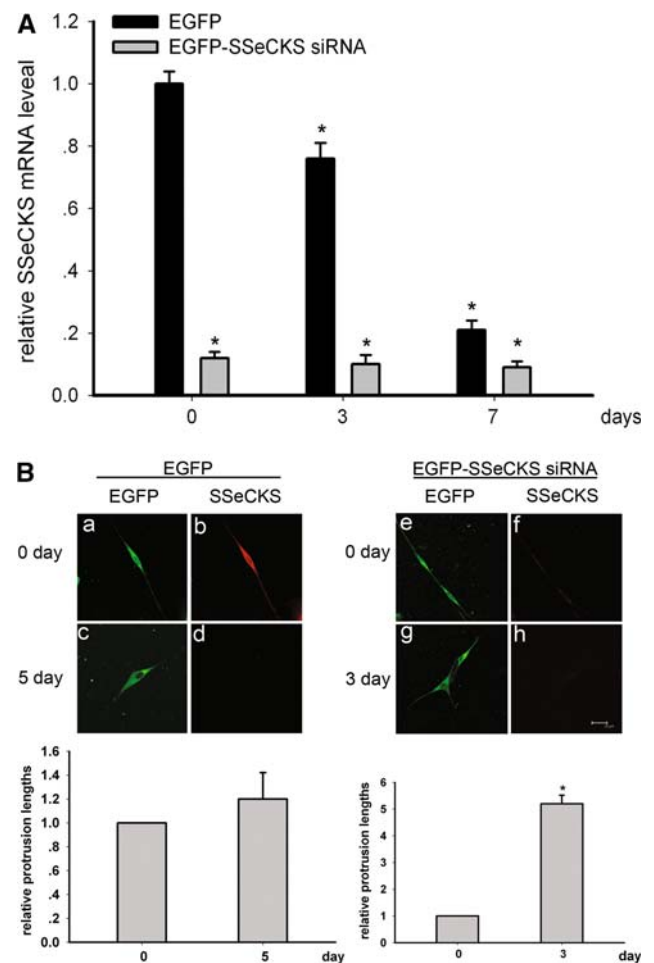


Fig. 2 Long-term suppression of SSeCKS expression enhances cAMP-induced morphological changes. **a** PT-PCR analysis showed a significant down-regulation of SSeCKS mRNA expression in SSeCKS siRNA transfected Schwann cells treatment with cAMP at day 0, 3, and 7. GAPDH expression was used as a reference. Black bars, Lenti-EGFP-infected cells (EGFP); gray bars, Lenti-EGFP-SSeCKS siRNA-infected cells (EGFP-SSeCKS siRNA). * indicated significant differences compared with the EGFP group, $P < 0.001$ (*t* test, $n = 3$). **b** Lenti-EGFP-SSeCKS siRNA-transfected Schwann cells started to change their morphology after 3 days treated by cAMP, but no morphology changes observed in control lenti-EGFP-infected Schwann cells until day 5. Average protrusion lengths were quantified and data are mean values \pm SEM. * $P < 0.001$ (*t* test, $n = 6$). Scale bars: 20 μ m

in SSeCKS-suppressed Schwann cells treated with 10 μ M cAMP for 3 days, when it was merged in uninfected Schwann cells after 7 d (Fig. 3a). NGFRp75 expression which demonstrated the lacking of myelin protein, decreased earlier in SSeCKS knockdown cells when exposure to cAMP (Fig. 3b).

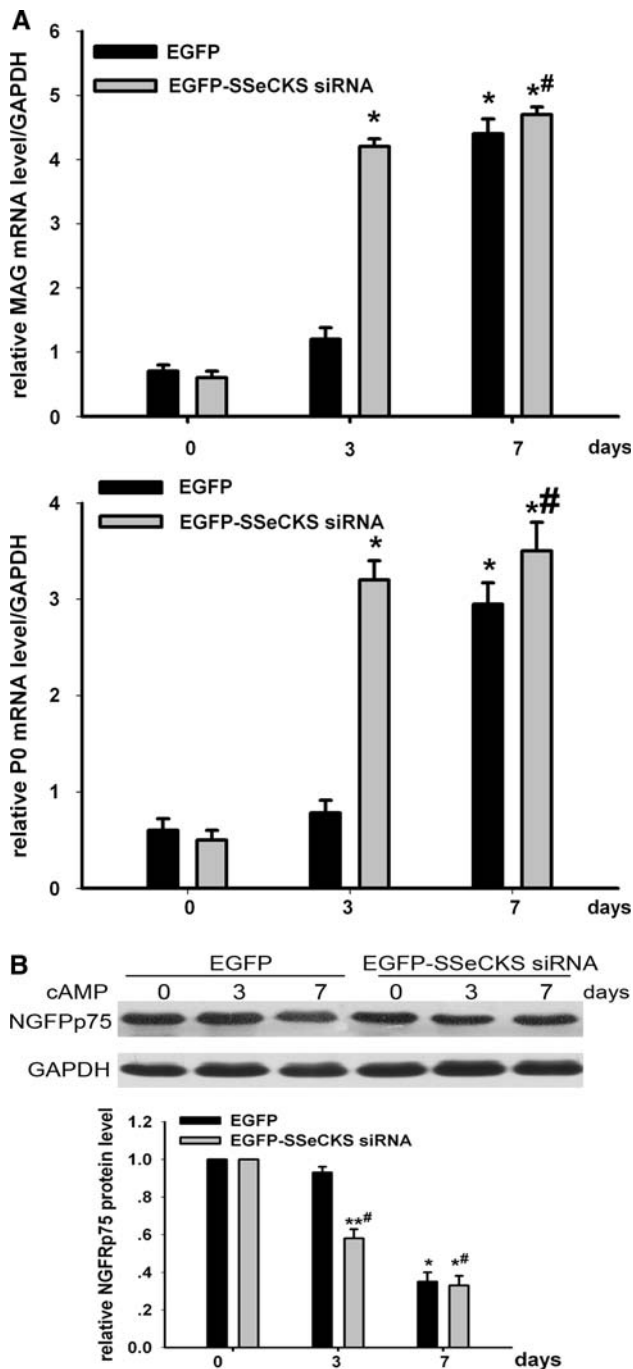


Fig. 3 Suppression of SSeCKS expression in Schwann cells accelerates cAMP-induced myelin induction. **a** RT-PCR revealed that the expression of P0 and MAG were induced in SSeCKS-suppressed cells by cAMP treatment for 3 days while it was merged in control-infected Schwann cells after 7 days treatment. GAPDH expression was used as a reference. * $P < 0.001$, compared with the EGFP group and # $P < 0.001$, compared with the EGFP-SSeCKS siRNA group (t test, $n = 3$). **b** Western blot analysis indicated that NGFRp75 was reduced in SSeCKS-suppressed Schwann cells after 3 days cAMP treatment, and in control Schwann cells by 7 days treatment. GAPDH expression was used as a reference. * compared with the untreated EGFP group, $P < 0.005$. ** compared with the 3 d cAMP-treated EGFP group, $P < 0.001$. # compared with the untreated EGFP SSeCKS-siRNA group, $P < 0.005$ (t test, $n = 3$)

Suppression of SSeCKS Leads to Accelerated In Vitro Myelination of DRG Axon

SSeCKS suppression was shown to promote Schwann cells maturation, therefore we investigated whether the myelination process was affected. To test this, co-cultured Schwann cells with DRG cells were then fixed and processed for anti-MBP immunofluorescent staining after co-culturing for 14 days. The result showed that more MBP formation in SSeCKS-transfected Schwann cells compared to control lenti-EGFP vector-transfected Schwann cells (Fig. 4a). Western blot confirmed the result (Fig. 4b).

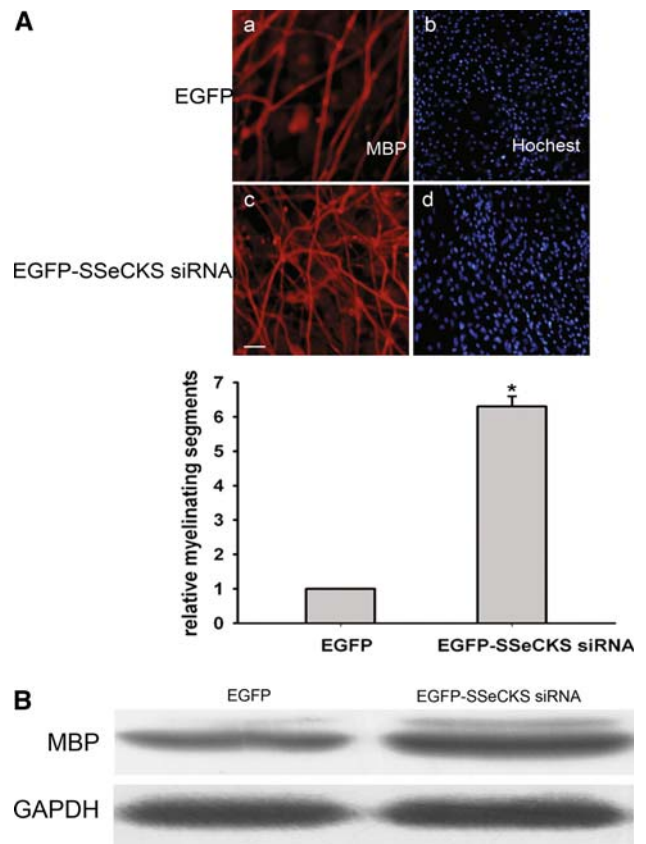


Fig. 4 In vitro myelination of DRG axons with SSeCKS-suppressed Schwann cells. **a** Immunostaining revealed Schwann cells formed MBP (red) positive myelination when seeded on DRG co-cultures for 14 days. Hoechst were used to label nucleus. The lengthen of MBP-positive segments in EGFP-SSeCKS siRNA Schwann cells and DRG co-culture were more than the EGFP-transfected. Data represent mean \pm SEM. * $P < 0.001$ (t test, $n = 3$). Scale bars: 10 μ m. **b** Western blot analysis revealed that SSeCKS knockdown promoted MBP expression earlier in Schwann cells and DRG co-culture: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

SSeCKS Suppresses Schwann Cells Differentiation and Myelination via PI3K-Akt Pathway

It has been identified that Akt phosphorylation plays an important role in Schwann cells differentiation and myelination [8]. Our study has proved that by the application of LY294002, an inhibitor of phosphatidylinositol-3-kinase (PI3K) pathway (Fig. 5a, b). Recent studies have documented that SSeCKS could suppress Akt serine 473

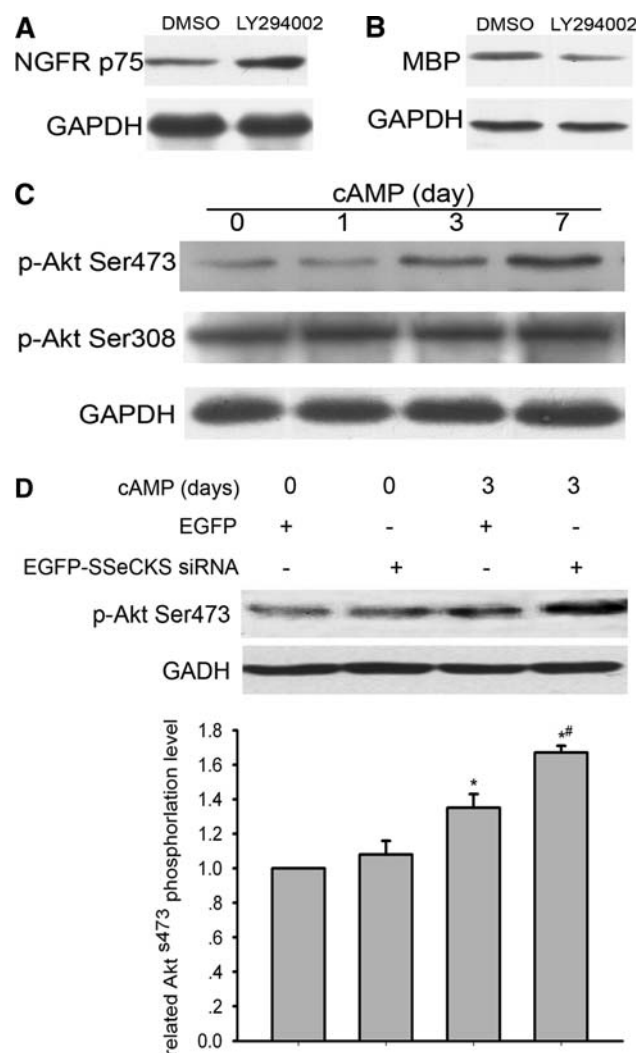


Fig. 5 SSeCKS promotes Schwann cells differentiation via the suppression of Akt phosphorylation. **a** LY294002 reduced cAMP-induced NGFRp75 reduction. **b** LY 294002 inhibited MAG expression in Schwann cells and DRG co-culture. **c** The phosphorylation of Akt^{S473} (p-Akt Ser473) was increased in Schwann cells differentiation process induced by cAMP, but the phosphorylation of Akt^{T308} (p-Akt Tyr308) remained unchanged. **d** In SSeCKS knockdown Schwann cells, p-Akt Ser473 was increased by cAMP treatment for 3 days, comparing with the control EGFP-transfected Schwann cells. Average pixel intensity for each band was normalized to GAPDH, and differences were given as * compared with the untreated EGFP group, $P < 0.005$. # compared with the cAMP-treated EGFP group, $P < 0.005$ (t test, $n = 3$)

(Akt^{S473}) phosphorylation via affecting PI3K signaling [23]. We measured the Akt^{S473} phosphorylation in cAMP-treated Schwann cells. Similar to previous results, we found that the phosphorylation of Akt^{S473} was greatly increased after treatment for 3 days, and it reached peak at day 7. However, the phosphorylation of Akt tyrosine 308 (Akt^{T308}) remained unchanged (Fig. 5c). To determine the relationship between SSeCKS and Akt in Schwann cells differentiation, the lenti-EGFP-SSeCKS siRNA was used to knockdown SSeCKS expression. After 10 μ M cAMP treatment for 3 days, phosphorylation of Akt^{S473} in lenti-EGFP-SSeCKS siRNA-transfected Schwann cells was significantly increased compared to control lenti-EGFP vector-treated Schwann cells (Fig. 5d).

Discussion

In the present study, we demonstrated that SSeCKS expression was down-regulated during Schwann cells differentiation. Silencing SSeCKS expression accelerated cellular differentiation, myelin genes expression, and in vitro myelination. SSeCKS suppressed the phosphorylation of Akt^{S473}, which plays a critical role in Schwann cells differentiation, resulting in inhibition of Schwann cells differentiation. In conclusion, SSeCKS is an inhibitor of myelination and schwann cells differentiation, which down-regulation is a prerequisite to allow differentiation progress.

Cell differentiation is delicately regulated by diverse extrinsic stimuli as well as by intrinsic programs in multiple tissue and organs [24]. Elevation of intracellular cAMP in Schwann cells acts as an instructive signal for their differentiation into a myelinating phenotype. However, the effects of cAMP on cultured Schwann cells are complicated and not well understand. Although originally shown to promote proliferation [25, 26], increasing the cyclic nucleotide has also been shown to induce Schwann cells differentiation. In the initial reports of cAMP-mediated differentiation, neonatal rat Schwann cells were treated with higher concentrations of cAMP analogs (0.5–3 mM) [9]. Numerous reports have demonstrated either a proliferation response or a differentiation response to cAMP based on its levels. Differentiation requires a higher concentration of cAMP [27], proliferation and cyclin D1 induction are associated with lower concentration of cAMP [28]. More recently, these disparate effects of cAMP have been attributed to the presence of growth factors and/or serum. Increasing cAMP alone, in the absence of serum or exogenous growth factors, does not increase proliferation but induces formation of differentiation markers, such as P0 [11]. However, the synergistic effect and robust cell proliferation are seen when higher

concentration of cAMP in combination with serum or growth factors such as neuregulin are used [29].

Protein kinase A is a down stream effector of cAMP, which is activated on the nucleotide binding to the regulatory subunit of the kinase. SSeCKS is a protein kinase A anchoring protein. It has one sequence which is the 1,537–1,563 of gravin in its carboxyl terminus [15]. The residues of gravin form the PKA-anchoring site, as a peptide made from this sequence is sufficient to block binding of the RII subunit of PKA in the overlay assay, and protein fragments containing this region bind RII with nanomolar affinities. However, much recent work has indicated that PKA signaling cross talks at different levels along with receptor tyrosine kinase (RTK)-induced pathways exerting either a positive or a negative effect on RTK downstream signaling [30, 31]. Because different RTKs use many common effectors, such as Ras-Raf-MEK-ERK and PI3K-Akt, an action of PKA on any of the possible intermediates might subvert the requirement for a specific growth factor or change important properties of the transduction mechanism. The following study has shown that SSeCKS suppressed Akt^{S473} phosphorylation via affecting PI3K signaling in prostate cancer [23]. Our results indicated that in the absence of SSeCKS, cAMP-induced Akt activation was inhibited in Schwann cells. The possible explanation was that the expression of SSeCKS might inhibit the activation of Akt via the suppression of PKA cascade.

As a member of cell cycle inhibitor, SSeCKS is a primary candidate as a Src inhibited gene, which will interfere with the G1/S transition of the cell cycle [32]. Regulated cell cycle exit is imperative for Schwann cells differentiation from precursors to myelinating cells [33]. In traditional way, knockdown cell cycle inhibitor expression will promote the cell cycle. But more and more studies have shown that knockdown of these gene expressions will promote cell differentiation. It has been reported that knockdown p57kip2 and p27 expression could induce Schwann cells differentiation, in vitro myelination and cell cycle exit. But these effects have not been proved in p15, p16, p18, and p19 [33]. So the mechanism that knockdown SSeCKS expression would not reduce differentiation and myelination needs to be further investigated.

In our experiment, we discovered that SSeCKS inhibited Schwann cells differentiation and myelination by affecting a number of important processes such as morphology, and myelin expression. Knockdown SSeCKS in Schwann cells significantly promoted myelination in DRG–Schwann cells co-cultures. In Schwann cells differentiation and myelination, SSeCKS inhibited the phosphorylation of Akt^{S473} to prevented myelination progress. But why the cycle inhibitor plays an inhibition role during myelination has not been determined.

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